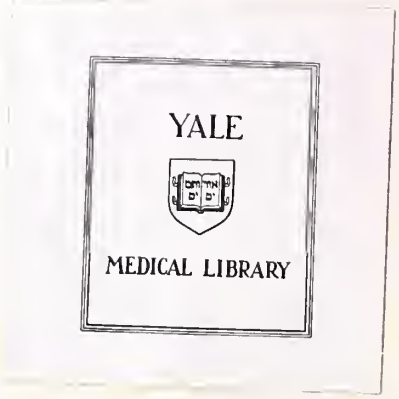


Permission for photocopying or microfilming of "HUMAN PLATELET MONOAMINE OXIDASE AND ~~EARLY~~ PRIMARY CHILDHOOD AUTISM"

(TITLE OF THESIS)

for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.

Gerson Marc Steinstein

Signature of Author

MAY 22, 1978

Date

HUMAN PLATELET MONOAMINE OXIDASE

AND

PRIMARY CHILDHOOD AUTISM

GERSON MARC STERNSTEIN

YALE UNIVERSITY SCHOOL

OF MEDICINE

MAY, 1978

SUBMITTED AS FULFILLMENT OF

THESIS REQUIREMENT FOR

THE DEGREE OF DOCTOR OF

MEDICINE

TABLE OF CONTENTS

INTRODUCTION

Monoamine Oxidase (MAO) has proven to be most problematic for both basic scientists and clinicians. The review of the literature is organized in terms of the techniques used to elucidate the molecular basis of MAO's activity, hoping that some understanding can be distilled from an extensive and conflicting research history.

I would like to acknowledge the help offered to me by Dr. Gerry Young, Professor C. Norman Gillis and Mary Ellen Kavanaugh. Their concern was appreciated, their good spirits indispensible. For Donald Cohen, I have the profoundest gratitude. For me, the most important aspects of the past year in research had little to do with laboratories and platelets, as Donald well knows. Finally, I thank my wife for her love manifest in her endurance that supported me during this work.

PHARMACOLOGY OF MAO

Neuropsychiatric interest in Monoamine Oxidase (MAO, EC 1.4.3.4.) was stimulated by several major findings in the 1950's. Zeller discovered that iproniazid, an anti-tuberculosis agent, was a potent in vitro and in vivo inhibitor of MAO. During the same period, Amin, Vogt and Bertler found that Serotonin (5-HT), Norepinephrine (NE), and Dopamine (DA), all substrates for MAO, were distibuted in the brain.

Clinically it had been noted that tuberculous patients treated with iproniazid experienced a lightening of their mood. This observation led Loomer and Saunders to introduce iproniazid for the treatment of depression. Thus the groundwork was laid for the "catecholamine hypothesis" of affective disorders postulated by Schildkraut and Kety in 1965.

During the 1960"s, the investigation of MAO inhibitors was remarkable for the repeated finding that the substrate could influence the activity of the inhibitor. Differences in inhibition kinetics, pH optima, Michaelis constants, ion effects and substrate inhibition suggested the presence of either multiple enzymes or one enzyme with multiple catalytic sites. Since all the work was done with reversible inhibitors on non-purified enzyme preparations, it was not possible to rule out steric factors as an explanation. in addition, no inhibitor was found to selectively inhibit MAO activity to one substrate and not another.(for review, see Achee, Gabay and Tipton, 1977; Youdim & Collins, 1975)

Johnston, in 1968, introduced clorgyline as an irreversible inhibitor of MAO prepared from rat brain (Johnston, 1968). The concentration of drug required to inhibit the oxidation of 5-HT was several orders of magnitude less than that necessary to inhibit benzylamine (BZ) oxidation. Of most significance was the effect of clorgyline on tyramine (TYR) oxidation. Only about half of the MAO activity toward TYR was inhibited by clorgyline concentrations completely inhibitory of 5-HT oxidation. The rest was inhibited at concentrations necessary to stop BZ oxidation. This bi-phasic dose-response curve, with a plateau at medium inhibitor concentrations (Fig. 1), led Johnston to postulate the presence of two distinct enzyme species. One clorgyline sensitive and the other clorgyline resistant, designated Type A and Type B respectively. 5-HT was the preferred substrate for Type A and BZ preferred for Type B (Hall, Logan & Parsons, 1969), with TYR a common substrate.

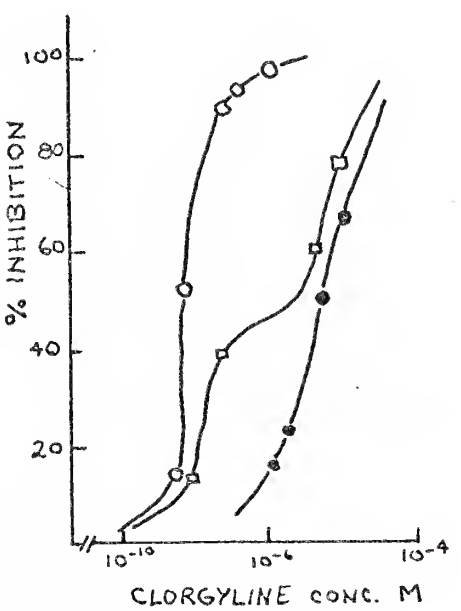


Fig.1
(Fowler, et. al., 1978)

o - 5-HT
□ - TYR
● - BZ

Since Johnston's report, other irreversible inhibitors have been introduced. Some have characteristics resembling clorgyline, while others selectively inhibit Type B MAO. Most notable of the later are Deprenyl (Knoll & Magyar,1972) and Pargyline (Fuller, 1970).

Johnston's work serves as the paradigm for studying the selectivity of MAO inhibitors and the proportions of MAO types in various organs from various species, always extrapolating from data on rat brain and liver. Selectivity experiments are performed assuming 5-HT oxidation to be exclusively by Type A and BZ oxidation by Type B. If an inhibitor shows concentration differences in its inhibition of 5-HT versus BZ oxidation, it is assigned a Type A or B selectivity depending upon which substrate is inhibited by the lower concentration. Many of the reports on the selectivity of reversible inhibitors have used a single concentration of several substrates against a single concentration of inhibitor. A difference in "% inhibition" is purported to suggest selectivity. However the differing K_m values of the substrate could account for these results. Consequently, to effectively suggest selectivity of reversible inhibitors, it is necessary to compare inhibition of substrates at concentrations that are equal percentages of their K_m's. Conclusive evidence for selectivity requires a full kinetic study determining both the type of inhibition

and the K_i value (i.e. concentration of inhibitor at which enzyme activity is reduced by 50%). (Houslay, Tipton & Youdim, 1976)

In studying the properties of MAO from different organs and species, the assumption is often made that they will be similar to rat brain and liver in substrate specificity, differing only in the relativeproportion of Type A to B. A "biphasic" or "double-sigmoidal" curve of % MAO activity to a common substrate like TYR versus inhibitor concentration implies substrate oxidation by two enzyme forms. The relative proportion of Type A to B is assessed by estimating the height of the plateau joining the sigmoidal parts of the curve.

Much recent work hase suggested that the simple binary classification Of MAO extrapolated from rat studies may not be universally applicable. For example, ox heart studies show a biphasic inhibition of 5-HT, suggesting that 5-HT is a substrate for both types, (Mantle, et. al., 1976). Pig brain MAO is reported to be Type B, but it is active toward 5-HT (Squires, 1972; Tipton & Spires, 1968). Lyle and Callingham haveshown that the apparent biphasic effect of clorgyline to BZ oxidation in the rat heart is due to contamination by a connective tissue or blood vessel amine oxidase differing from MAO,(Lyle & Callingham,1975). This points to a disadvantage in using simple tissue homogenization for MAO preparation.

Substrate and inhibitor specificity can best be studied in terms of kinetic parameters. Supersaturating levels of substrates are frequently used in studying MAO properties. If a so-called Type A substrate is oxidized, it is assumed that Type A must be present. In fact, Type B has been found to metabolize Type A substrates and vice versa (for review, Fowler, et. al., 1978). Substrate specificity is best determined by comparing K_m's and reaction velocities at substrate concentrations at equal percentages of their K_m (Jain, Sands & Von Korff, 1973).

Ekstedt studied the specificities of Type A and Type B MAO prepared from intact rat liver mitochondria. Type A substrates (5-HT and NE), Type B substrates (BZ and 2-phenylethylamine, PEA) and common substrates (TYR and Tryptamine, TRYP) were used in mixed-substrate experiments. In addition, the B form was selectively studied by inhibiting the A form with clorgyline, and the A form studied by using deprenyl to inhibit the B form (Ekstedt, 1976).

Mixed-substrat experiments in uninhibited mitochondria showed that all six substrates inhibited activity to radiolabelled 5-HT to the same extent. This implies that the K_m of each substrate to the A form are equivalent. The substrates inhibited the activity to radiolabelled PEA, though not to the same extent. This suggests differences in the K_m of each substrate to Type B MAO.

Lineweaver-Burke plots performed on deprenyl-treated and clorgyline-treated mitochondria revealed competitive inhibition of all substrates for both A and B MAO. Deprenyl-treated mitochondria still retained 4% of activity to PEA that could be inhibited by low concentrations of clorgyline. Similarly, the 2% activity to 5-HT remaining after clorgyline treatment was inhibited by low concentrations of deprenyl. Thus, 5-HT can be oxidized by Type B MAO and PEA can be oxidized by Type A MAO. The K_m of 5-HT for MAO A was 6×10^{-5} M versus 2.5×10^{-3} for MAO B. Interestingly, the K_m of PEA for MAO A was also 6.2×10^{-5}, the same as 5-HT, but PEA's K_m for MAO B was 4×10^{-6} M. Substrate specificity is affected solely by the differing affinities of the two substrates for MAO B.

It has been found that high concentrations of either clorgyline or deprenyl will give 100% inhibition of both MAO A and MAO B. (Neff & Yang, 1974). Hence, substrate and inhibitor specificity, though significant at physiological concentrations, will be obscured by experiments performed at pharmacological levels.

Jain has criticized the use of irreversible selective inhibitors as supporting evidence for multiple enzyme forms (Jain, 1977). Pointing out that pargyline, deprenyl and clorgyline all inhibit MAO by stoichiometric binding to the FAD prosthetic group (Edmondson & Singer, 1976),

the multiple form hypothesis based on differential inactivation would require some other molecular basis other than similarity of binding to the flavin moiety.

Tipton showed that at high clorgyline concentrations, rat liver MAO was inhibited to 5-HT, BZ, and TYR. However, inhibition of 5-HT oxidation was more rapid than BZ. TYR oxidation inhibition occurred in two phases, corresponding to the fast and slow components for 5-HT and BZ respectively. Tipton concluded that clorgyline selectivity was caused by differing rates of attack (Tipton, 1971).

Mantle, Wilson and Long introduced a new compound, PCO, with clorgyline-like inhibitor characteristics (Mantle, Wilson & Long, 1975a). In experiments using intact rat liver mitochondria, PCO inhibition was found to be time-dependent. After 45 minutes of preincubation, 5-HT inhibition was 100%, with activity toward TYR only reduced 50%. An hundred-fold increase in PCO concentration gave 50% inhibition [illegible] without preincubation. It was concluded that PCO differential sensitivity arises from different rates of attack at two centers of oxidation. The rate of attack against the PCO-sensitive site was 200 times faster than the attack at the insensitive site. Interestingly, PCO was a competitive inhibitor of TYR oxidation with hyperbolic kinetics. Hyperbolic competitive kinetics suggests competitive inhibition at two sites by an inhibitor with different affinities

for the two enzyme sites (Mantle, Wilson & Long, 1975b). Still, these experiments cannot discriminate between one enzyme with different environments, two distinct enzymes, or one enzyme with two sites.

The time-dependent inhibition by clorgyline was further investigated by Lyles and Greenawalt. Many of the experiments studying the pharmacologically separable forms of MAO use preincubation periods differing from author to author. This could alter the characteristics of reported enzyme sensitivity. Using intact rat liver mitochondria the effects of preincubation time and enzyme concentration were studied (Lyles & Greenawalt, 1977).

For 5-HT, time-dependent inhibition was observed only with clorgyline concentrations equal to 2×10^{-10} M and low enzyme amounts. At clorgyline concentrations greater than 2×10^{-7}M, only the second half of the biphasic curve to TYR oxidation was affected by preincubation. It seemed that only MAO B was sensitive , an observation confirmed by preincubation effects at all inhibitor concentrations against BZ oxidation.

The experiments point out that some molecular basis defining the rate of attack at a catalytic site , regardless of the final mechanism of MAO inactivation at the flavin moiety, does exist. Careful attention to pre-incubation time, assay duration and protein content must be paid when

comparing data from inhibitor studies of MAO.

Lineweaver-Burke plots of substrates in the presence of varying concentrations of inhibitor permit classification of inhibition kinetics. Changes in the slope, intercept or both, reflect competitive, uncompetitive and non-competitive (mixed) inhibition respectively. Mantle, et. al. showed that multiple-substrate experiments with 5-HT, NE, DA, TYR, TRYP, BZ, PEA all resulted in competitive kinetics for rat liver (Mantle, Wilson & Lonz, 1975b).

Substrate competition experiments can be an effective technique for studying the relationship between differing enzyme activities. If two substrates are metabolized at the same active site, they will be competitive inhibitors of each other. When two active sites are present with exclusive specificity, the presence of one substrate should have no effect on the activity toward the other.

By setting the concentration of one substrate at its K_m, and the inhibiting substrate at twice its K_m, the inhibition should be 50% if the substrates compete for the same site, and no inhibition if independent sites are involved. If two substrates share the same site, the K_i of a substrate should equal its K_m. Under these conditions, using adult human brain and liver MAO of intact mitochondria, White and Wu found that 5-HT and PEA do not inhibit MAO activity to each other. This indicates that these substrates

are catalyzed by independent sites. Experiments with other substrates were not as clearcut (White & Wu, 1975).

The interpretation of data derived under the conditions of these experiments requires certain assumptions that may not be appropriate. If either substrate is metabolized by additional active sites, the per cent inhibition will depend on the affinity and specific activity of the other sites. Another requirement is that allosteric changes of multiple enzyme forms do not occur. Finally, the experimentally derived K_m values used for adjusting substrate concentrations could be the average of the affinities for multiple active sites. This has been shown to be true for rat liver mitochondria (Ekstedt, 1976; Mantle, Wilson & Long, 1975a).

Competitive kinetics can be classified into linear and non-linear types by replotting the slopes derived from Lineweaver-Burke plots against inhibitor concentration (Piszkiewicz, 1977). The slope of such replots reflects the effect of an inhibitor at an active site. Replots that are linear signify either that there is competition at only one site, or that multiple sites are independent and are kinetically equivalent for substrate and inhibitor. Non-linear replots signify competition at multiple sites or allosteric effects on a single site. They are designated either hyperbolic, with a convex-upward curve, or parabolic, with a concave-upward curve.

By extrapolating the initial and final slopes of non-linear replots to the inhibitor concentration axis (abscissa), two apparent K_i's reflecting high and low modes of inhibitor binding are obtained. Hyperbolic plots show high affinity binding at low inhibitor concentrations, while parabolic plots show high affinity binding at high inhibitor concentrations. (Fig. 2)

Computer simulation of the competitive inhibition of two independent active sites kinetically similar for the substrate but with two differing affinities for the inhibitor yields hyperbolic kinetics (Mantle, Wilson & Long, 1975b). Parabolic replots cannot be derived from such a model, and are evidence for an interacting site model of enzyme activity.

In all mixed-substrate experiments that show competitive inhibition, the inhibition is in effect a dilution of the labelled product of the substrate by the unlabelled products of the competitive inhibitor. This can be shown by double-label experiments. Consequently, the K_i is equivalent to the K_m of the inhibitor at an active site. The fit between experimentally derived K_m's and K_i's for substrates and their correlation to computer derived kinetic parameters will differentiate between hyperbolic kinetics from two independent sites or two interacting sites.

Mantle, Wilson andLong found linear kinetics for all substrates versus 5-HT oxidation suggesting one site of MAO activity for 5-HT. Linear replots were obtained for most substrate interactions. However, 5-HT was found to inhibit the "common substrates" DA and TYR by hyperbolic kinetics. The good correlations between K_m's and K_i's experimentally, and to simulated plots point to the existence of two independent active sites for MAO oxidation (Mantle, Wilson & Long, 1975b). This is in agreement with work on rat liver showing two K_m's for 5-HT differing by two orders of magnitude (Ekstedt, 1976).

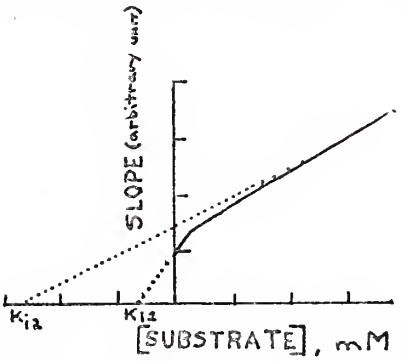


Fig. 2. Computer simulation of hyperbolic slope replot for competitive inhibition of 2 kinetically equivalent sites for substrate by an inhibitor with different affinities (K_{11}= high affinity; K_{12}= low affinity) for two independent sites. Dotted lines represent extrapolations. (Mantle, Wilson and Long, 1975b)

MULTIPLE FORMS-ELECTROPHORESIS

The heterogeneity of MAO activity revealed by pharmacological studies prompted the implementation of electrophoretic techniques for elucidating the structural basis of this behavior.

In 1967, Youdim and Sandler used polyacrylamide gel electrophoresis to separate different constituents of solubilized MAO from rat liver and human placenta (Youdim & Sandler, 1967). Soon after, Kim and D'Iorio used cellulose acetate electrophoresis to describe multiple bands of MAO activity from rat liver homogenates. In addition, they reported similar results when solubilization by detergent was compared to sonication (Kim & D'Iorio, 1968). Kim and D'Iorio were not willing to preclude either incomplete solubilization or enzyme association with subparticles of membrane debris as explanations for the multiple bands.

Youdim followed his initial work with experiments to characterize the properties of the electrophoretic bands. Briefly summarized, it was found that K_m's for substrates were similar, yet the V_{max}'s were not. The bands showed differing inhibitor sensitivities, differing pH optima for maximal kynuramine (KYN) oxidation, and differing susceptibility to heat inactivation (Youdim, Collins & Sandler, 1970). The results were similar for both rat liver and brain (Collins, Sandler, Williams & Youdim, 1970). The molecular weights (MW) of the bands were equivalent,

290,000-320,000, by gel filtration. With ultracentrifugation, the bands had a MW= 150,000 (Youdim & Collins, 1971).

The denaturation of partially purified rat liver MAO with 8M urea or 1% sodium dodecyl sulfate (SDS) yielded a single band upon electrophoresis. The band had 100% loss of activity, and a molecular weight of 78,000. Reassociation by dialysis resulted in 5 bands on electrophoresis, similar to the non-denatured preparation. When each isolated band of activity was subjected to denaturation, a single band of MW= 76,000 migrated identical to the band of denatured whole enzyme preparation. However, upon reassociation and electrophoresis, each original band of activity resulted in 5 bands with a pattern identical to the denatured-reassociated whole enzyme preparation (Youdim & Collins, 1971).

Youdim and Collins interpreted their results as revealing an MAO consisting of inactive subunits of MW = 78,000. they concluded that MAO could not be an enzyme of MW = 150,000 with two sub-units, nor an enzyme with four sub-units and a MW = 300,000. They only entertained the possibility of two dissimilar sub-units, one with a substrate binding site (Ss), and one without (S). Thus, an enzyme with two sub-units would permit only two active forms (SsS and 2Ss), and an enzyme with four sub-units would give only four active forms (4Ss, 3SsS, 2Ss2S, Ss3S). Neither model gives

the five active forms achieved by electrophoresis. Instead the authors felt the five active forms could represent conformational enzymes, differing only in tertiary structure. This would also also explain how the denaturation-reassociation of each individual form could yield all five forms (Youdim & Collins, 1971). It should be noted that the authors assumed that the five forms are not artefacts of their solubilization procedure. Nor did they entertain other models of sub-unit interaction in an enzyme. For example, two inactive sub-units of MW = 75,000 may be necessary to bind with the FAD cofacter to yield an active enzyme with MW = 150,000.

Sierens and D'Iorio pointed out the problems inherent in the harsh solubilization techniques. Using the ionic detergent deoxycholate and gel electrophoresis, they were able to separate two fractions of MAO activity from rat liver mitochondria (Sierens & D'Iorio, 1970). One fraction acted on BZ and 5-HT, the other on BZ alone. However, compared to untreated mitochondria, both fractions had reduced activity. MAO activity to 5-HT was only 2% and to BZ but 20% in the first fraction. Further experimentation showed that dialysis partially prevented the loss of activity. Deoxycholate bound to enzyme could not explain the results as it did not migrate with either fraction.

The authors concluded that the apparent MAO multiplicity

was likely secondary to irreversible damage to 5-HT activity, and not to MAO isoenzymes differing in protein constitution. This experiment pointed out both the need for a solubilization method that causes only a minor reduction in activity as tested against several substrates, and that chromatographic or electrophoretic separations alone are not sufficient for establishing enzyme multiplicity.

In later work, Diaz, Sierens and D'Iorio were able to partially separate 5-HT activity from BZ activity using density gradient electrophoresis and sonication with only a 20-25% reduction of MAO activity to BZ, 5-HT and KYN. The results were suggestive of at least two different activities, though the data could not distinguish between two enzymes, multiple forms of one enzyme, or association of enzyme with non-protein molecules (Diaz-Borges & D'Iorio, 1972)

Oreland and Olivecrona studied the binding of MAO to mitochondria and found that the liberation of soluble MAO enzyme correlated with the extraction of cardiolipin, an anionic phospholipid. Using purified phospholipids, they found soluble complexes of MAO and cardiolipin, or insoluble complexes if the cardiolipin were bound to delipidated mitochondrial membrane residues (Oreland & Olivecrona, 1971; Olivecrona & Oreland, 1971).

Tipton explored the relationship between separate activities of MAO and bound membrane material by measuring the phospholipid content of electrophoretically separable forms of MAO (Tipton, 1972). Phospholipid content and mobility were inversely related, with the fastest moving band having the lowest amount of phospholipid. Reasoning that if lipids were responsible for apparant isoenzymes, then the distribution of electrophoretic forms should be affected by techniques that extract lipids. Organic solvents caused a significant loss of activity, but the non-ionic detergent Triton X-100 yielded only a single band with mobility similar to the fastest moving band.

Tipton stated that Triton X-100 had no effect on MAO activity to BZ, 5-HT, DA or TYR, using purified rat liver MAO. He did not report his results, and it is difficult to rule out the possibility of damage to one enzyme form that could be obscured by measuring activity against a non-separated preparation.

Tipton did find that prolonged dialysis against Triton X-100 or Phospholipase A resulted in the loss of MAO activity, though a single band was still observed. This raised the possibility that lipid is vital to the stability of MAO, and that the loss of electrophoretic heterogeneity could result from the denaturation of active forms.

Oreland showed that 1.5% Triton X-100 for three hours

did not affect the K_m of BZ, compared to a mitochondrial preparation (i.e. enzyme in situ) of pig liver MAO, but the V_{max} was reduced , in agreement with Tipton.

To circumvent the methodological issue raised by detergent binding to enzyme, Oreland used an organic solvent and salt solubilization-purification procedure with a yield of 20% MAO activity. Both the K_m and V_{max} were lowered for BZ, corroborating the influential role played by lipids (Oreland, 1972).

Oreland did not feel that his extraction procedure may have preferentially lost one of the isoenzymes, though it could account for the change in Michaelis parameters versus the enzyme in situ. For example, the loss of a form with a higher K_m for BZ that significantly contributes to BZ oxidation at the BZ concentration used, could appear as a decrease of K_m and V_{max}.

Oreland concluded this was not the case because the percentage activities to various substrates versus activity to TYR were not significantly changed. Such experiments are carried out at a single substrate concentration significantly higher than its K_m to ensure enzyme saturation. The activity recorded is then considered the V_{max}. Consequently, the loss of one form would be observed only if it were responsible for a significant portion of enzyme activity at that substrate concentration.

Pharmacological experiments with pig liver found only the B form, so Oreland's work is not conclusive (Squires, 1972). In later experiments with rat liver, Oreland discovered the preferential loss of Type A activity with retention of the B form (Ekstedt & Oreland, 1975).

Houslay and Tipton continued the investigation of protein-membrane interactions and rat liver MAO by using sodium perchlorate, a chaotropic agent. These compounds are capable of weakening hydrophobic bonds between proteins and lipids, thus disrupting protein-membrane complexes.

Polyacrylamide gel electrophoresis was used to compare a perchlorate-treated solubilized enzyme preparation, an untreated solubilized enzyme preparation, and a mitochondrial outer membrane preparation that contained 90% of enzyme activity. Both the outer membrane preparation representing MAO in situ, and the perchlorate-treated enzyme showed one band of activity migrating similar to the slowest moving band of the untreated enzyme. In addition, one band of activity originally described by Youdim was found to be an artifact of the gel loading procedure.

Sodium perchlorate treatment caused no loss of activity for incubation periods up to ten minutes. There was no difference in the K_m or V_{max} for BZ, TYR or DA, suggesting

that perchlorate treatment did not alter the active site of the enzyme. All substrate-related differences in heat inactivation, pH optima, and inhibitor effects of harmine, phenylethylhydrazine and clorgyline were abolished by perchlorate treatment (Houslay & Tipton, 1973).

Houslay and Tipton concluded that the apparant electrophoretic heterogeneity and substrate specific activities of MAO were probably secondary to the solubilization procedure. The electrophoretic heterogeneity did not exist in the mitochondrial outer membrane preparation, though none of the other parameters of heterogeneity were tested. They speculated that the vigorous sonication-detergent steps could result in unknown complexes of membrane lipid, enzyme and Triton X-100 causing apparant multiple forms.

It is not possible to categorically deny the existence of multiple forms since the solubilized enzyme preparation retained less than 50% of the original activity. In this context, it should be recognized that Houslay and Tipton used 2% Triton X-100, previously found to inactivate MAO (Tipton, 1972; Oreland, 1972), and 50 mM Tris buffer, a reversible non-competitive inhibitor of TYR, 5-HT and PEA oxidation with a K_i of 15 - 25 mM (Fowler, Callingham & Houslay, 1977). BZ is not inhibited by Tris. Consequently, all experiments exploring substrate-specificity are most difficult to interpret.

Singer has warned that all chaotropic agents affect protein structure. While catalytic and inhibitory prroperties may be intact, the more subtle and significant in vivo regulatory properties can be modified (Singer, 1976).

Jain and Sands reviewed the literature on electrophoretic heterogeneity and found that all such reports used extended periods of sonication for their enzyme preparations. By using a non-sonication procedure for solubilization of human brain MAO, they found only a single band of activity on polyacrylamide gel electrophoresis. In addition, the Michaelis constants for this purified enzyme were very similar to those of an intact mitochondrial enzyme preparation. Thus Jain and Sands added evidence to the artefact theory of MAO electrophoretic multiplicity, implicating sonication (Jain and Sands, 1974).

The non-sonication procedure used by Jain and Sands was originally developed by McCauley and Racker. This purification used 100mM Tris and 1.5% Triton X-100 (McCauley & Racker, 1973). The Michaelis constants were derived from a solution of 100 mM potassium phosphate buffer, a significant inhibitor of MAO (Jain, Sands & Von Korff, 1973; Van Woert & Cotzias, 1966). These constants were very similar to those reported for the B form of human brain MAO (White & Glassman, 1977).

Sonication does not invariably lead to electrophoretic

heterogeneity, as studies on the human platelet have revealed (Collins & Sandler, 1971). Sandler and Youdim contended that sonicationwould have been expected to give an infinite gradation of membrane particles rather than the reproducible bands (Sandler & Youdim, 1972). It has been pointed out that specific sites in the protein structure may exist at which it will preferentially split or dissociate under the stress of sonication or solubilization (Jain, 1977).

All of the work referred to above on multiple bands of MAO activity used a colourimetric technique for localizing MAO activity with nitroblue tetrazolium reduction by MAO as the marker. However, some MAO substrates themselves can reduce tetrazolium (NTZ) salts (Lagnado & Sourkes,1958), and NTZ strongly inhibits MAO activity as compared to radioassay techniques (Lagnado, Okamoto & Youdim, 1971). It is also possible that one of the enzyme forms is not MAO, though it can oxidize monoamines to give electrons to NTZ yielding a positive reaction (Guha & Ghosh, 1970). The existence of such an enzyme with activity toward TRYP and inhibited by iproniazid has been detected in the brain of rats (Ghosh & Guha, 1978).

Diaz-Borges and D'Iorio used the non-ionic detergent 12.5% Lubrol and sonication to prepare rat liver MAO and 0.4% Lubrol in solutions for polyacrylamide gel

electrophoresis (Diaz-Borges & D'Iorio, 1973). Using a radioassay, they achieved a complete separation of BZ and 5-HT activity with overlapping activity toward TYR.

Upon comparing the radioassay to NTZ staining, little correlation was seen. 5-HT, TYR or TRYP in the staining mixture gave a broad purple band that was abolished by pre-incubation with the MAO inhibitor isocarboxid. No such band was observed with BZ as the substrate. Staining of the gels with BZ, TYR or TRYP gave a yellowish-purple backround with some zones lighter or darker, giving the appearance of bands. However, isocarboxid pretreatment failed to abolish this effect, suggesting it was not due to MAO activity.

These experiments point out the inadequacies of colourimetric staining for detection of MAO activity. They are remarkable for the total independence of electrophoretic mobility of BZ and 5-HT activity, analogous to Johnston's model.

The solubilization technique used had previously been shown to give 83% recovery of BZ activity and 79% recovery of 5-HT activity, so the possibility of selective degradation of one form is less likely (Diaz-Borges & D'Iorio, 1972). Experiments on the separate activities assessing phospholipid content, MW, activity after chaotropic treatment, and pharmacologic, immunologic and kinetic

characteristics are necessary to establish the existence of two distinct enzyme proteins.

Inoue, Robinson and Dost compared radioassay and colourimetric assays using rat liver and brain prepared according to Youdim in 1.5% Triton X-100. Additionally, the preparation was dialyzed against phosphate buffer for over 60 hours to remove the Triton X-100 (Inoue, Robinson & Dost, 1976). Where four bands were found on gel electrophoresis with NTZ, only one band (corresponding to the least mobile one with NTZ) had activity with a C^{14}-labelled substrate. The results were the same using radiolabelled BZ, TRYP or TYR. Consequently, the Triton X-100 per se does not cause the heterogeneity observed by Youdim, though all work using NTZ must still be viewed with caution.

MOLECULAR WEIGHT OF MAO

The molecular weight of MAO has been studied to elucidate the nature of the multiple activities. In general, the MW has been measured by various investigators with little agreement. Only if the molecular weight is calculated per mole of flavin, the enzyme co-factor, is there agreement - 1.2 - 1.5 x 10^5 g MAO per mole of 8α - (cysteinyl)FAD (Sandler & Youdim, 1972).

Youdim and Collins, using a 1.5% Triton X-100 preparation of rat liver MAO found a MW = 300,000 for both whole enzyme and its electrophoretically separated forms using Sephadex gel filtration. However, ultracentrifugation gave a MW = 150,000. 8 M urea or 1% SDS yielded inactive sub-units with MW = 75,000 by gel filtration. By using phenelzine, an irreversible MAO inhibitor that binds to the flavin moiety, Youdim found a molar ratio of Phenelzine / FAD / 150,000 MW (Youdim & Collins, 1971).

Oreland, using a ketone extraction method, obtained a purified MAO with a strong tendency to aggregate. A series of gel filtrations on Sephadex G-200 with increasing concentrations of the ionic detergent sodium cholate, gave a MW = 290,000 for enzyme still active to BZ in 2.5% cholate. 10% cholate showed two peaks of activity, one at 290,000 and another at 115,000. When the enzyme of MW = 115,000 was rechromotographed without cholate, it aggregated and appeared in the void volume. Using pargyline,

Oreland found a molar ratio of 1 : 1 : 1 of pargyline/ flavin/ protein of MW = 115,000 (Oreland, 1972). Oreland interpreted these results to suggest protein aggregation as the basis for electrophoretic heterogeneity. An alternative interpretation is that the sodium cholate may have affected the retention on the column, giving falsely decreased estimations of MW.

Shih and Eiduson solubilized rat brain MAO in Triton X-100 and used both agarose and Sephadex electrophoresis to separate MAO. Agarose separation yielded two fractions, A and B (not to be confused with Johnston's pharmacological designations) with MW=1.5×10^6d and 500,000 respectively. To investigate the question of aggregation, the MAO was rechromotographed in the presence of 8M urea, speculating that A would disappear, with a concomitant increase in B. In fact, some of A did disappear, but all of B seemed to disaggregate to a fraction C with a smaller MW. All fractions were active to 5-HT, BZ, TRYP and PEA. Rechromatography of B alone showed partial reaggregation to A. Further, when low concentrations of enzyme preparation were added to the agarose column, some of A and all of B converted to C. Sephadex G-200 chromatography yielded analogous results.

In general the specific activity to substrates increased with separation, i.e. C>B>A, though the change was not

consistent through the steps. This differential increase in specific activity was interpreted as evidence for forms of MAO that differ in teh aggregation of sub-units (Shih & Eiduson, 1974).

Experiments by Inoue, Robinson and Dost suggest another explanation. Noting the wide use of Triton X-100 in solubilization procedures, they pointed out that dialysis to remove the detergent from the partially purified enzyme is rarely carried out (Inoue, Robinson & Dost, 1976).

If dialysis were for less than 60 hours for rat liver and brain MAO, a significant amount of MAO activity was held up on Sephadex G-200. Most came off in the void volume, in agreement with Oreland and Shih and Eiduson. The portion held up corresponded to Fraction B. Dialysis for longer than 60 hours showed a single peak of activity appearing in the void volume.

Inoue felt this phenomenon could be due to the high affinity of the protein to the Triton X-100 with stabilization of the enzyme in a disaggregated form. As little as 0.3% Triton X-100 added to enzyme dialyzed for 60 hours and already passed through the column, caused the complete retardation of enzyme activity on Sephadex G-200. Consequently, variable gel filtration behavior used to show multiple forms and to determine the MW can be partially attributed to differing amounts of detergent remaining in the enzyme.

IMMUNOLOGICAL APPROACHES

The contraversy over multiple forms led some investigators to employ immunological techniques in the investigation of enzyme heterogeneity. In general, the degree of structural-relatedness of MAO forms was studied using antigen-antibody cross-reactivity in double diffusion experiments.

Hartmann and Udenfriend used bovine liver separated into three fractions with MAO activity (Hartmann & Udenfriend, 1972). Two of the forms were pure protein, designated C_1 with MW=400,000 and 4 moles FAD per mole, and C_2 with MW=1,200,000 and 12 moles FAD per mole. The third form was a protein fraction contaminated by other proteins and residual Triton X-100, designated Fraction A.

Antibodies were induced against C_2 because it would presumably contain the most antigenic sites with some differing from C_1 and Fraction A. Immunodiffusion yielded single precipitin lines without spurs, suggesting both antigenic identity and the absence of sites on C_2 not found on the other forms. The impurity of Fraction A left the possibility of an MAO antigenically distinct from C_2. Immunoprecipitin titration resulted in the total removal of MAO activity from Fraction A, ruling out the presence of a structurally unrelated form.

C_2 antibody tested against a crude Triton X-100 solubilized liver MAO gave 93% removal of MAO activity. The preparation retained only 65% of total liver MAO activity to BZ, so the possibility of a denatured form exists.

Cross-reacting C_2 antibody to bovine brain MAO left immunoprecipitin lines of identity without spurs. Yet, immunotitration could precipitate only 80% of brain MAO activity to BZ. The remaining 20% was felt to represent a "brain-specific MAO" antigenically distinct.

It would be interesting to explore these immunologically separable forms with other substrates and inhibitors using proper kinetic techniques, and to see if antibody to the "brain-specific MAO" could be induced.

McCauley and Racker extended the work on bovine brain and liver MAO antigenicity, though their antigen preparattion differred from Hartmann and Udenfriend (McCauley & Racker, 1973). They added the use of selective inhibitors and more substrates to characterize brain MAO activity not precipitable by antibody induced against liver MAO.

Essentially, they found that non-precipitable MAO had activity to 5-HT and NE much greater than its activity to BZ. Precipitable MAO had no activity to 5-HT or NE, while retaining significant activity to BZ. Both forms had activity to DA and TYR. The non-precipitable activity toward NE and 5-HT was inhibited by harmaline, an MAO A inhibitor, and resistant to pargyline inhibition. The precipitated MAO showed the reverse inhibitor sensitivity.

These results are analogous to Johnston's model, so McCauley and Racker concluded that non-precipitable brain

MAO was indeed Type A MAO, a form antigenically distinct from bovine liver MAO, designated Type B.

Unfortunately, the authors did not attempt immunotitration or the addition of anti-globulin to the supernatant to rule out incomplete precipitation secondary to soluble antigen-antibody complexes. Therefore , it is still conceivable that the two pharmacologic types differ only in the number of similar sub-units.

Dennick and Mayer prepared rat and human liver MAO specifically purified by prolonged 1.5% Triton X-100 incubation to electrophoretic homogeneity in the presence of SDS. Activity to 5-HT was completely lost, and only 3% of total activity was retained (Dennick & Mayer, 1977).

Antibody induced from this MAO preparation was able to immunoprecipitate 100% of the activity to 5-HT, BZ and TYR in liver mitochondrial extracts by equal volumes of anti-serum. Dennick and Mayer concluded that all liver MAO activity resides in one macromolecular species.

This conclusion is legitimate only if the specificity for a substrate is absolute. The relative activities of the liver mitochondrial extracts suggest that MAO A, with its activity toward 5-HT greater than its activity to BZ, was not present. Inhibitor studies of the mitochondrial extracts might have proven illuminating. The extended Triton X-100 treatment used in their preparation could

have preferentially denatured an A type MAO more dependent on lipid moieties for activity.

Youdim has shown that SDS will yield a homogeneous protein from active multiple forms, that is inactive with a MW one-half to one-quarter that of active MAO. Consequently, Dennick and Mayer may have formed antibody to an inactivated MAO A, though not antigenically destroyed.

All the immunologic studies are equally consistent with a single enzyme modified by membrane-binding masking the antigenic response (Tooze, 1973), or the presence of more than one enzyme.

ONTOGENIC PATTERNS

The ontogenesis of MAO has been studied in many organs of many species (for review, Gripois, 1975). Eiduson the rat brain, liver and heart using polyacrylamide gel electrophoresis and NTZ staining. He found differences between adult and neonatal rats in the number and position of bands, and that testosterone could affect the number of bands (Eiduson, 1972). Blatchford, Holzbauer and Youdim found that the specific activity to TYR and DA increased with age in the hypothalamus and striatum of three rat strains. TRYP oxidation decreased in the hypothalamus and striatum from only two of the strains, suggesting age, species and substrate effects on the development of MAO activity (Blatchford, Holzbauer & Youdim, 1975).

Jourdikian attempted to classify maturational rates of Type A versus Type B MAO in the mouse brain. Type A, identified by using 5-HT as a substrate and clorgyline as an inhibitor, reached adult levels by 20 days old, when Type B was only 50% adult values (Jourdikian, Tabakoff & Alivisatos, 1975).

In the rat, fetal liver has a fully developed binary system of MAO. However, fetal brain MAO was 97% Type A. It is not until Day 45 post-natally that the adult proportions of 60% A : 30% B is achieved (Mantle, Garrett & Tipton, 1976). Mantle speculated that Type A may represent a fetal form since it is the predominant type in glioma

and neuroblastoma (Donnelly, Richelson & Murphy, 1976).

Using a different strain of rats, Bourgoin discovered adult levels of MAO B by Day 20, while MAO A activity was 80% higher in brain stem homogenates and 20% higher in forebrain homogenates at Day 20 post-natally, as compared to adult rats. Kinetic analysis revealed a higher V_{max} in neonatal rats with no difference in the K_m of 5-HT, implying greater amounts of enzyme in the pup. At birth, rat brain 5-Hydroxyindole acetic acid (5-HIAA) is equal to adult 5-HIAA, though 5-HT at birth is only 33% of adult levels (Bourgoin, et.al., 1974; Bourgoin, et. al., 1977). Thus maturational changes in MAO activity are significant for the regulation of brain monoamines.

Experiments in the ontogenesis of MAO have not discriminated between changes with age of brain lipids, mitochondria or protein molecules. They do bring evidence pointing to the importance of the distinction between MAO activities, each under different regulation.

MAO IN SITU - THE MITOCHONDRION

The work of Schnaitman and Greenawalt has localized MAO to the outer membrane of the mitochondrion. The outer membrane (OM) can be dissociated from both the matrix (M) and inner mitochondrial membrane (IM) by digitonin treatment. The small vesicles formed from OM were found to have a greatly increased specific activity for MAO and represented a large fraction of the total mitochondrial MAO activity. The IM-M fraction, called mitoplasts, retain essentially no MAO activity (Greenawalt, 1972).

Mitochondrial membranes are dynamic structures with their components undergoing continuous turnover. The IM and OM proteins undergo independent, asynchronous synthesis (Gear, 1970). 20% of mitochondrial protein is in the IM, 70% in the matrix, 6% as intermembrane protein, and only 4% in the OM (Schnaitman & Greenawalt, 1968). as a result, changes in the ratio of IM to OM, or OM to mitochondrion could cause changes in the amount of enzyme activity per amount of protein. The IM : OM ratio in rat heart is 20 : 1, while in liver it is 5 : 1. This could explain the relatively lower MAO activity of heart mitochondria (Greenawalt, 1972). Differences in phospholipid to protein ratios and in the lipid composition of the two membranes have been reported (Ernster & Kuylenstierna, 1970).

This intimate localization of MAO in the OM forms the basis for much of the dispute regarding enzyme multiplicity. Membrane-bound enzymes have a tendency to change characteristics upon solubilization and purification. These changes are referred to as allotopic properties. Houslay and Tipton found that solubilization of MAO causes a significant alteration in the kinetic mechanism. It implies the necessity for the enzyme to adopt a conformation normally dictated by the membrane environment (Houslay & Tipton, 1975). Perchlorate treatment abolished the biphasic inhibition pattern of both deprenyl (Tipton, Houslay & Mantle, 1976) and clorgyline to TYR oxidation (Houslay & Tipton, 1973).

The mitochondrial environment could enforce specific conformations on one protein, preferentially stabilize one of several proteins, or expose different catalytic sites on one protein. Any of these alternatives would present multiple enzyme activities in vivo.

The allotopic properties of MAO suggest that the microenvironment plays a significant role in MAO activity, especially in creating a relatively non-polar environment to favor amine oxidation. Some investigators have studie MAO in purified intact mitochondria in an attempt to resolve inconsistencies and to elucidate the action of the enzyme in a more natural state.

Gabay used a mitochondrial preparation from bovine brain with a fair degree of morphological integrity and freedom from contamination as assessed by electron microscopy and enzyme marker studies. This preparation had 80% of the MAO activity found in brain homogenates (Gabay, Achee & Mentes, 1976).

Substrates were tested against pH, ions and thermostability, all expected to affect environmental conditions. Cationic species seemed to have no effect, but phenylalkylamines (PEA, TYR)were more affected by phosphate ions, and the indolealkylamines were more susceptible to inhibition by chloride ions. Increased chloride gave non-competitive inhibition to the indolealkylamines and mixed-competitive inhibition to the phenylalkylamines. This might indicate a difference in binding groups for the substrates within an active site.

Gabay also reported differing pH optima for the K_m and V_{max} of the substrates. There have been studies implying

that the true substrate for MAO is the unprotonated amine (McEwen, et. al., 1968; McEwen, et. al., 1969; Williams, 1974). Upon correcting for the degree of ionization, Gabay found that the the optimal pH for the Michaelis constants of the unionized amines was pH= 7.4. Finally, there was no difference in thermostability to any substrate in intact mitochondria, though this has been reported as evidence for the existence of multiple forms.

Clorgyline and harmaline were found to be selective for 5-HT activity versus PEA, but dose-response curves were biphasic for all substrates. The Type B inhibitor deprenyl required pre-incubation to give substrate selectivity at low inhibitor concentrations. The difference stemmed from the slower rate of PEA inactivation. This was true for high clorgyline concentrations also.

Mixed-inhibitor studies were most suggestive of two sites distinct for different inhibitors though not mutually exclusive for substrates. The combination of Type A and Type B inhibitor had additive effects to achieve total MAO inhibition, whereas two inhibitors of the same type gave only partial inactivation (Achee & Gabay, 1977).

Of interest was the finding that 100% inhibition of 5-HT was never achieved by these authors, though activity to all other substrates could be totally abolished. Williams found 5-HT oxidizing activity localized in the

circumventricular structures of rat brain that is not inhibited by high clorgyline concentrations (Williams, Gascoigne & Williams, 1975). Though substrate and inhibitor studies have yet to be done, these results are clinically significant for the suggestion of an MAO type intimately associated with the hypothalamus and CSF distinct from the activities normally studied.

Improvement in subcellular fractionation using density and zonal centrifugation have led to the conclusion that mitochondrial populations are heterogenous. Differences in bouyant density and enzyme activity have been reported. In brain, it has been possible to separate neuronal and glial mitochondria, and synaptosomal and non-synaptosomal mitochondria (for review, Van den Berg,1973).

The first report of mitochondrial heterogeneity of MAO activity was by Kroon and Veldstra, using homogenization of rat brain in isotonic sucrose to separate out synaptosomal mitochondria. Zonal centrifugation in sucrose density gradient gave a partial separation of synaptosomes and free mitochondria. The distribution of enzyme activities suggested that MAO activity to 5-HT and DA is localized in mitochondria differing from NE and KYN oxidizing MAO. (Kroon & Veldstra, 1972).

High sucrose densities cause marked mitochondrial and synaptosomal distortion through osmotic shifts, so Youdim

used an iso-osmotic Ficoll-sucrose gradient to prevent artefacts (Youdim, 1974). Rat brain and liver showed heterogeneity of MAO activity to TYR, TRYP, KYN and DA, though no fraction had activity exclusive for any substrate.

The MAO activity as measured by Youdim and Kroon and Veldstra were using substrates at saturating concentratins. All of Youdim's substrates are "common" as defined by Johnston pharmacologically. The heterogeneity could represent differences in the proportion of MAO A to MAO B in the mitochondria. In this vein, Neff used sucrose density gradients and found two regions of activity to TYR. Using the ratio of PEA-MAO activity to 5-HT-MAO activity, it was found that mitochondria with higher bouyant density seemed to have MAO B by virtue of its high PEA:5-HT oxidizing ratio (Neff, Yang & Fuentes, 1974).

Owen used a Ficoll-sucrose discontinuous gradient to separate three populations of rat brain mitochondria. Two were derived from synaptosomes, and a third ostensibly non-synaptosomal and glial (Owen, et. al., 1977). The synaptosomal populations had equal specific activity to 5-HT, but all three groups differed in specific activity to PEA. The heavy synaptosomal fraction (SM2) had greater PEA-MAO activity than the light (SM1) population. The free mitochondrial population had the lowest specific activity to both substrates.

Clorgyline and deprenyl gave biphasic inhibition of TYR oxidation in all fractions. The plateaus suggested that SM1 had a greater proportion of MAO A than did SM2. SM1, with the lowest PEA:5-HT activity ratio was most susceptible to clorgyline and least sensitive to deprenyl. The reverse was true for the SM2 fraction.

These findings were extended to mouse lung mitochondria separated into a light population with low PEA:5-HT activity ratio and a denser population with high PEA:5-HT activity ratio (Gallagher, 1977). When the mouse was pretreated with pargyline (MAO B inhibitor) before sacrifice, 5-HT MAO activity remained high and peaked in the light mitochondrial fraction, while PEA oxidation was almost completely blocked. Pretreatment with clorgyline strongly inhibited 5-HT oxidation, while activity to PEA was unaffected, and peaked at the high density fraction.

The use of lung mitochondria avoid contamination with membranous material found in synaptosomal preparations. These results confirm the separation of substrate-specific MAO activity in heterogenous mitochondrial populations. It is interesting that brain and lung seem to have MAO B in a denser mitochondrial population. One could speculate that differing lipid:protein ratios may be involved.

Student and Edwards added sub-synaptosomal fractionation to the studies of rat brain and found no selective

localization of either type MAO on the synaptic plasma membrane (Student & Edwards, 1977). Consequently, the difference in Type A to Type B MAO proportions seem to be inherent in the mitochondria themselves. Synaptosomal mitochondria seem to have more MAO A and less MAO B relative to non-synaptosomal mitochondria.

Upon comparing the specific activity of synaptosomal MAO to whole brain homogenates, the ratio of MAO B to MAO A in the synaptosomes was 43% that of the homogenates ratio, reflecting a doubling of synaptosomal MAO A specific activity. Free mitochondria and homogenate had the same ratio.

The ratio of MAO B to MAO A in synaptosomes from cerebellum was 400% the ratio in cortex. Homogenates of these regions gave only a 30% difference. These results demonstrate that the use of brain homogenates to assess MAO activity can obscure differences in activity at the significant cellular location - the synapse.

LIPIDS AND MAO HETEROGENEITY

After Tipton demonstrated that electrophoretic mobility was inversely related to phospholipid content (Tipton, 1972), and that chaotropic treatment caused a loss of electrophoretic heterogeneity (Houslay & Tipton, 1973), it became important to explore the role of lipids in the binary system of MAO generated from pharmacological data.

The biphasic inhibition by Clorgyline and Deprenyl of rat liver MAO activity to TYR is abolished by chaotropic treatment (Houslay & Tipton, 1973; Tipton, Houslay & Mantle, 1976). Eluting a solubilized enzyme from DEAE-cellulose with Triton X-100 also caused a loss of the selective response to inhibitors (Houslay & Tipton, 1975). MAO activity became less sensitive to clorgyline and more sensitive to deprenyl, suggesting a loss of MAO A activity by delipidation.

Youdim studied the inhibition of the multiple electrophoretic forms of rat liver MAO by clorgyline. Clorgyline inhibition of both DA and KYN oxidation was inversely related to mobility (Youdim, Holzbauer & Woods, 1974). Coupled with the inverse relation of mobility to phospholipid content, the idea arises that the absence of lipid decreases clorgyline sensitivity. Youdim found this to be the case in cat brain MAO (Youdim & Holzbauer, 1976). Williams and Lawson studied the class of compounds, propargylamines, of which clorgyline is a member, and

found that inhibitor potency correlated with lipophilicity (Williams & Lawson, 1975).

Theoretically, lipids could account for multiple activities in one protein by stabilizing one active site versus another, or, one configuration of the site versus another configuration. If the second case were true in MAO, one would expect the transformation of the A activity into B activity upon delipidation with an increase in activity to B form substrates. Ekstedt and Oreland tested this using delipidated rat liver mitochondrial membranes with MAO still bound (Ekstedt & Oreland, 1975). The B-type activity was recovered completely, but no A-type was retained. When the B-form was inhibited with deprenyl before delipidation, and the membrane-bound MAO then extracted, no MAO activity was observed. Thus no B activity was produced from transformation of A-type activity by delipidation. On the other hand, clorgyline pretreatment had no effect on MAO B activity.

White and Glassman applied a variety of approaches to elucidate the effects of solubilization techniques on the microenvironment of MAO. The properties of MAO in intact mitochondria were compared to MAO solubilized with a 30-50% yield by 3% Triton X-100. With low concentrations of either deprenyl or clorgyline, it was possible to selectively determine the Michaelis constants of substrates to each

type of MAO activity. Solubilization did not change the K_m's of 5-HT or PEA and the ratio of activities to the substrates were similar. The sensitivity to inhibitors was unchanged and biphasic curves to common substrates were preserved (White & Glassman, 1977). Therefore both types of MAO activity were relatively intact after solubilization.

Double-labelled mixed-substrate experiments can assess Type A and Type B activity in the same incubation mixture. In intact human brain mitochondria, PEA and 5-HT are oxidized independent of each other, suggesting at least two active sites corresponding to MAO A and MAO B (White & Wu, 1975). It was not possible to separate the activities by molecular size using gel filtration, or by differences in net surface charge as determined by electrofocusing. These results indicate that different MAO sites could be part of the same macromolecule (White & Glassman, 1977).

A variety of treatments were found to preferentially inactivate 5-HT oxidation without affecting PEA MAO activity. Solubilization itself caused a slight decrease in the 5-HT : PEA activity ratio. Incubation in SDS, an ionic detergent, and sonication decreased MAO A activity and inhibitor selectivity. At low concentrations, sulfhydryl reagent caused a selective A-site inhibition in both MAO preparations, preventable by high levels of 5-HT , but not PEA. Thiols and anionic phospholipids could protect the

A-site in the solubilized MAO from inactivation by either high salt concentrations or prolonged incubation without substrate (White & Glassman, 1977).

The significance of this work lies in its presentation of evidence for two independent active sites resulting from differences in both protein conformation and lipid environment. The A-site has easily oxidized sulfhydryl moieties, and is highly susceptible to membrane perturbations. Veryovkina was able to inhibit MAO A activity to 5-HT by blocking 3 sulfhydryl groups per 1 x 10^5 grams of protein, while B-site inhibition required the blocking of 5 groups (Veryovkina, Abdel Samed & Gorkin, 1972).

Houslay prepared a model to test the role of lipids in the selectivity of clorgylin and deprenyl. Pure Ca^{2+}-Mg^{2+}ATP-ase from rabbit sarcoplasmic reticulum can be prepared in various lipid bilayers to give protein surrounded by a phospholipid annulus. By choosing the components, one can provide either a fluid or rigid lipid microenvironment for the enzyme at a given temperature. The activity of the ATP-ase is lowest at temperatures that make the lipid annulus rigid, and is maximum in fluid lipid environments. The effects of molecules on the fluidity/rigidity of the annulus can be sensitively monitered by changes in ATP-ase activity.

At temperatures that force a rigid annulus of the

phospholipids dimyristoyl lecithin (DML) and dipalmitoyl lecithin (DPL), clorgyline and deprenyl were able to activate the ATP-ase, while increasing bilayer fluidity. Dose-response curves revealed that clorgyline affected activation and fluidization at concentrations two orders of magnitude less than deprenyl. The difference in potency could not be explained by the small differences in the partition coefficients for the drugs (Houslay, 1977).

This model suggests that the inhibitor selectivity is not a protein-mediated event, but stems from the differing potencies for lipid perturbation. As clorgyline is a Type A inhibitor, the model supports the hypothesis that the A-site is more lipid-dependent for its activity.

Huang, Eiduson and SHih introduced the use of electron spin resonance (ESR) techniques to study MAO. Spin-labelled p-hydroxyamphetamine (SHA), a reversible MAO inhibitor, was used as a probe of the activity of a partially purified , solubilized rat brain MAO (Huang, Eiduson & Shih, 1976).

A characteristic signal is quenched when SHA is bound to the enzyme. The change in signal is proportional to changes in the enzyme-inhibitor complex, so quantification of enzyme-probe and enzyme-substrate interactions is possible.

When SHA concentration was plotted against enzyme-inhibitor

concentration, the probe was found to bind to MAO in a triphasic curve, felt to represent three different affinity sites for SHA in the non-homogenous enzyme preparation.

ESR is particularly good for studying the active site of an enzyme in situ. Huang and Eiduson identified and characterized three binding sites by intensive and extensive parameters (Huang & Eiduson, 1977).

Each binding site has a specific affinity that is a function of temperature according to the Gibbs-Helmholtz relationship:

$$\Delta H^{\circ} = -R \frac{d \ln K}{d \frac{1}{T}}$$

where ΔH° is the apparant heat of formation of the site-SHA complex; K is the apparant association constant; R is the gas constant, and T is temperature. The slope of ln K versus $\frac{1}{T}$ will give the heat of formation (ΔH°) at each binding site.

When ln K was plotted against 1/T, three straight lines of different slopes were observed, characterizing three distinct binding groups - α, β, γ. The intersection of the lines reflect the temperature at which one site predominates over another, called the transition temperature. The transition temperature is associated with changes in the lipid domain of biomembranes that have been found to correlate with changes in the functional activity of

membrane-bound proteins.

Pretreatment with clorgyline and deprenyl caused preferential inhibition of the binding sites, with α left after clorgyline and β left after deprenyl. This supports the concept of two sites differing in lipid microenvironment. The A site seems to be composed of β and γ, and the B site composed of α and γ. That both sites share γ supports the data that 5-HT and PEAcan be substrates at either site.

The role played by the lipid microenvironment of MAO in the intact mitochondrial outer membrane in the generation of substrate specific forms is of vital clinical importance. Any change of enzyme activity found to correlate with a specific clinical state could be interpreted in terms of either a change in environment or a change in MAO protein. Alternatively, changes in activity due to membrane alterations might go unnoticed if MAO studies are performed only on purified, solubilized enzyme.

IN VIVO EVIDENCE

The impetus for the study of the multiple forms of MAO originated in the hope that development of substrate-selective or tissue-selective inhibitors would lead to more useful tools for neurochemical research and to safe, clinically helpful psychotropic drugs. It was necessary, therefore, to study the effects of MAO inhibitors in vivo.

Collins, Sandler and Youdim compared the in vitro inhibition of the five bands of electrophoretically separable rat liver MAO, to inhibition of the same bands from rats pretreated with intrapeitoneal injections of tranylcypromine, pargyline and clorgyline (Collins, Youdim & Sandler, 1972).

The in vitro sensitivity of the bands to inhibitors varied widely with no correlation to the binary classification of Johnston. While distinct variation in the inhibition of the bands was found in vivo, these variations did not correlate with the apparent K_i's of each inhibitor to each band as determined in vitro.

The authors interpreted their results as evidence for the the in vivo significance of the electrophoretically generated forms of MAO, postulating that if the forms were artefactual, it might be expected that all forms would be inhibited to the same extent. Clearly, later research has proven the role played by phospholipids, Triton X-100 and NTZ staining

in the generation of these forms.

Yang and Neff demonstrated that rat brain MAO can be classified into the A/B system. Each type is responsible for the metabolism of different amines and can be preferentially blocked (Yang & Neff, 1974).

Intravenous clorgyline gave an increase in brain 5-HT, NE and DA with a concomitant decrease in 5-HIAA and 3,4-dihydroxyphenylacetic acid (DOPAC). Deprenyl had no effect on 5-HT or NE, but there was a significant increase in brain DA and decrease of DOPAC. Thus DA appears to be a substrate for both MAO A and B. This was substantiated both by biphasic inhibition curves to clorgyline and deprenyl in vitro, and by the observation that DA levels were greatest in the brain after pretreatment with both inhibitors.

The in vivo results correlated with the in vitro estimation of MAO inhibition using homogenized brain from pre-treated rats. The metabolism of injected PEA was found to be decreased by deprenyl, while unaffected by clorgyline.

Roth and Gillis elegantly confirmed these findings using perfused rabbit lungs. This system permits the accurate measurement of inhibitor, substrate and metabolite concentrations. Consequently, the pharmacokinetic problems of drug metabolism and distribution inherent in intact

animal studies are avoided while maintaining the advantage of an intact cell preparation (Roth & Gillis, 1975).

Roth and Gillis performed a douple-labelled mixed-substrate experiment with equimolar concentrations of H^3-5-HT and C^{14}-PEA perfused through the same lung and observed no competition of oxidation. The addition of harmaline, a reversible MAO A inhibitor, selectively decreased 5-HT oxidation without affecting activity to PEA, the results substantiate the in vivo significance of two separate enzyme activities.

The role of Type A and Type B MAO in the in vivo regulation of biogenic amines was studied using specific behavioral syndromes in the rat caused by increased synaptic 5-HT and DA respectively.

Green and Youdim reported that the 5-HT hyperactivity syndrome produced by tranylcypromine (MAO A and B inhibitor) and tryptophan load could not be induced by tryptophan loading and clorgyline pretreatment. The doses of clorgyline were shown to give 100% inhibition of 5-HT MAO activity in vitro. It was necessary to achieve 100% inhibition of both MAO A and B with clorgyline and deprenyl before the 5-HT behavioral syndrome could be induced by a tryptophan load. Clorgyline and tryptophan did not elevate brain 5-HT to the levels found after tranylcypromine or clorgyline + deprenyl (Green & Youdim, 1975). Analogous findings for

the behavioral syndrome produced by increased functional DA in the rat brain were reported (Green, Mitchell, Tordoff & Youdim, 1977). These authors found that the recovery of Type A MAO activity was 4,5 days and of Type B was 8,5 days. The rate of recovery from irreversible inhibition is generally thought to reflect the rate of new synthesis of enzyme. While the two forms seem to be under different regulation, one cannot discriminate among the alternatives of new protein synthesis, renewal of lipid moiety attachment, or different rates of specific mitochondrial regeneration.

Nonetheless,the experiments show that both 5-HT and DA can be metabolized by both types of MAO in vivo. It is possible that MAO A and B function as an integrated unit in the same cell (Houslay, Tipton & Youdim, 1976). Pathological states could arise from an imbalance between the two forms at a specific site.

HUMAN PLATELET MAO

The discovery of significant MAO activity in the human platelet is of particular significance for neuropsychiatric research, primarily due to the ease of obtaining samples for study. Platelet MAO can be helpful both diagnostically to characterize certain disorders, and therapeutically to assess the efficacy of drugs purported to act via alterations in MAO. Naturally, the degree of similarity between platelet MAO and MAO from other tissues, especially the brain must be determined.

Robinson compared rat liver to human platelet MAO activity towards BZ, TYR, TRYP and 5-HT. Though the specific activity was greater in rat liver, the rank order was the same. The K_m's for BZ and TYR were virtually the same, but those for TRYP and 5-HT were significantly different. Rat liver and human platelet MAO responded equivalently to MAO inhibitors (Robinson, et. al.,1968).

The authors felt the evidence pointed to similarities between the MAO's, though cross-species comparisons are difficult to assess. Of more importance was the good correlation found for the in vivo inhibition of platelet MAO and urinary excretion of TRYP. It appears platelet MAO is responsible for the peripheral metabolism of some biogenic amines.

Collins and Sandler prepared a solubilized MAO from human platelet mitochondria using two hours of sonication in the presence of 1% Triton X-100 and gel filtration

through Sephadex G-200. A single peak of enzyme activity with MW=235,000 was obtained, representing a 12-fold purification, though 90% of the original activity was lost (Collins & Sandler, 1971). The relative activities toward DA, TRYP, TYR, KYN and BZ did not change through various steps in the purification. A more labile form was not felt to have been lost. Unfortunately, neither 5-HT nor NE, substrates preferentially oxidized by MAO A, were tested.

The purified MAO showed a biphasic heat inactivation curve not apparant in MAO from lysed platelets, suggesting some change in the response to physical factors. The kinetic constants of substrates and inhibitors were in good agreement with those obtained by Robinson (Robinson, et. al., 1968).

Polyacrylamide gel electrophoresis using NTZ staining with TRYP as substrate resulted in a single band of activity. It is interesting to speculate why the identical purification technique used by Youdim to prepare his multiple forms in rat liver MAO yielded but one band in the platelet. This may reflect mitochondrial homogeneity in the platelet.

Amitriptyline, a tricyclic antidepressant, inhibits human platelet MAO in vitro (Edwards & Burns, 1974). The effect was equivalent for platelet sonicates, purified MAO preparations and perchlorate-treated enzyme, so the

inhibition was not due to membrane or lipid changes.

Amitriptyline inhibition is reversible for BZ, TRYP and PEA, but the mechanism was competitve for BZ and non-competitive for TRYP and PEA. The difference in kinetics led to the proposal that platelet MAO has two catalytic sites - one for BZ and another for TRYP and PEA.

Mixed-substrate experiments revealed that BZ and TRYP were mutually competitive, with PEA mutually non-competitive with BZ and TRYP. These results imply that BZ and TRYP share a site with another for PEA (Edwards & Chang, 1975). It is difficult to reconcile the two experiments with respect to inhibition of TRYP MAO activity.

Amitriptyline inhibition of platelet MAO was replicated in MAO B from human brain (Roth, 1976). When mixed-substrate experiments for BZ and PEA and MAO inhibition by amitriptyline were performed at high O_2 concentrations, all inhibition was found to be competitive. Thus, at high O_2 levels amitriptyline, BZ and PEA seemed to interact at one site.

The effect of O_2 is interpretable interms of the reaction mechanism. MAO possesses a "ping-pong" kinetic mechanism (Tipton, 1972; Yasunobu & Oi, 1972), as summarized by a two-step reaction:

(1) $E - F + RCH_2NH_2 \rightarrow EA \xrightarrow{H_2O} E'P_1P_2 \rightarrow E\text{-}FH_2 + NH_3 + RCHO$

$E + A \qquad\qquad E' + P_1 + P_2$

Deamination of Amine — Reduction of Flavin cofactor

(2) $E\text{-}FH_2 + O_2 \rightarrow E'B \longrightarrow EQ \rightarrow E\text{-}F + H_2O_2$

Reoxidation of flavin by oxygen

In such reactions, as the O_2 concentration decreases, the affinity for the substrate A increases (Tipton, 1972), protecting the reaction against variations in the O_2 level of its environment. The K_m for O_2 is about 0,250 mM, equivalent to the concentration of dissolved O_2 in air-saturated water at physiological temperatures. Both in vivo and in assay media, MAO will not be saturated by O_2. Consequently, the overall rate of reaction is dependent on the concentration and K_m of both O_2 and the substrate.

By raising the concentration of O_2 to super-physiological levels, Roth was able to saturate MAO and effectively keep it in the oxidized form. The deaminating step became rate-limiting so the competition between substrate and inhibitor could be observed. Kinetic analysis of the data revealed the theoretically expected linear competitive inhibition of BZ, PEA and amitriptyline at one site on the oxidized form of MAO (Roth, 1976).

These results indicate that mixed-substrate and inhibitor

experiments interpreted as evidence for multiple sites or forms must take into account the oxidative state of MAO. An inhibitor binding to either E or E' will give competitive or uncompetitive inhibition respectively. Non-competitive inhibition results from binding to both forms of the enzyme (Piszkiewicz, 1977). In the case of MAO, E corresponds to the oxidized form of the enzyme, and E' corresponds to the reduced form.

It is likely that both MAO A and B will exist in two conformations, one oxidized (E), and one reduced (E'). Binding to E' with lowered affinity at high inhibitor concentrations (i.e. second substrate or inhibitor like amitriptyline), will produce the hyperbolic replot data observed in the human platelet (Edwards & Burns, 1974) and human brain (Roth, 1976). The non-competitive kinetics result from both the decreased affinity to E', and the decreased rate of product formation. Possibly, the two conformations of platelet MAO reported by Kobayashi and Eiduson reflect changes in the oxidative state (Kobayashi & Eiduson, 1977).

Donnelly and Murphy utilized kinetic and inhibitor studies to characterize human platelet MAO as Type B. MAO was prepared by short sonication and freeze-thaw without attempts to purify and delipidate. The loss of labile or lipid-dependent forms is probably not a problem (Donnelly & Murphy, 1977).

Essentially 100% of MAO activity to TYR, DA, TRYP, PEA and BZ was inhibited by $1x10^{-6}$M deprenyl, while $1x10^{-7}$M clorgyline and 30 minute preincubation caused no inhibition. Both inhibitors gave single sigmoidal curves for common and B-site substrates. The K_m's for PEA and TRYP in the platelet are in agreement with those reported for human brain and liver MAO (White & Glassman, 1977). The evidence that platelet MAO corresponds to Type B very similar to MAO B found in other human tissues, especially the brain, is quite strong.

The homogeneity of human platelet MAO has particular significance for the question of enzyme multiplicity. Differential proportions of MAO A to MAO B have been shown to exist in many tissues of various species, though few preparations are exclusive for either type (for reviews, Achee, Gabay & Tipton, 1977; Fowler, Callingham, Mantle & Tipton, 1978). Among these experiments of nature are tissue culture preparations of rat C6 glial cells and mouse neuroblastoma N1E-115 cells, both MAO A, (Donnelly, Richelson & Murphy, 1976), and the human platelet. The discovery of a pure Type A human cell would afford the opportunity to determine the molecular basis of the distinction between MAO activities.

CLINICAL STUDY: MAO AND PRIMARY CHILDHOOD AUTISM

INTRODUCTION

Neuropsychiatric research on platelet MAO has been spurred by the repeated finding of reduced MAO activity in adult, chronic schizophrenia (Murphy & Wyatt, 1972; Meltzer & Stahl, 1974; Sullivan, Stanfield & Dackis, 1977), and bipolar depressive illness (Murphy & Weiss, 1972; Leckman, Gershon, Nichols & Murphy, 1977). Consensus on this topic is far from complete (White, Mcleod & Davidson, 1976; Owen, Bourne, Crow, et. al., 1976; especially the Correspondence section, New England J. of Med. 298(20):1150). Family and twin studies have suggested that reduced platelet MAO activity may be a biological marker for vulnerability to psychiatric disorders (Wyatt, et. al., 1973; Buchsbaum, Coursey & Murphy, 1976).

Comparison of the properties of platelets and serotonergic synaptosomes suggest that the platelet can serve as a model for the transport, storage, metabolism and release of 5-HT by CNS serotonergic neurons (Pletscher, 1968; Sneddon, 1973; Stahl, 1977). MAO plays a role in the uptake of moncamines of neurons (Trendelenburg, 1972) by altering their intracellular concentrations. This has not been studied as yet in the platelet, though it could help explain the elevated levels of 5-HT reported in a subgroup of autistic chidren (Hanley, Stahl & Freedman, 1977).

Primary childhood autism is a syndrome manifested during the first two years of life by disturbances in social, emotional and cognitive development (Rutter,1974). The aim of this study is to explore MAO activity in autistic children and their families to determine the occurence of a subgroup who either have variant levels of activity against normals, or are variant in relation to their family.

SUBJECTS AND METHODS

POPULATION

Twenty children (18 boys, two girls) with early childhood autism were the subjects of this investigation. They ranged in age from 4 to 23 years (mean=12.8 years). Nine children were unmedicated, six were on one drug and five were taking more than one. Aside from vitamins and antihistamines, medications included neuroleptics and anticonvulsants.

All children satisfied the diagnostic criteria for primary childhood autism: onset of symptoms of developmental disability before two years of age; absence or marked limitation of social relatedness; profound language delay and idiosyncrasies; need for environmental stability; movement disturbances; islets of better functioning; relatively normal motor landmarks.

Control subjects (15 male; 5 female) consisted of children (8 less than 15 years) and adults with no personal medical or psychiatric disturbances and no known family history of autism, aphasia, or other developmental disturbances.

Platelet MAO was assayed in the parents and normal siblings of nine children with autism to study the genetic contribution to MAO activity in these children.

METHOD

Consent was obtained from all participating individuals after explaining the purposes, procedures and risks of the study. Samples of blood for MAO assay from the index children were obtained as part of a broader initial evaluation and at subsequent occasions when the clinical indications for venipuncture were present.

Blood was obtained via standard venipuncture, using a 21-guage, siliconized needle, and collected in glass tubes containing 10.5 mg of EDTA. The blood was immediately placed on ice. Within three hours of collection, the platelets were harvested by centrifugation in succession at 175 and 300 g, each for ten minutes, and 4,000 g for twenty minutes to isolate the platelets. Platelets were washed in 2 ml of cold isotonic saline, reisolated by centrifugation, and stored as a pellet frozen for a maximum of two weeks.

Platelets disrupted by sonication were incubated in the presence of 1.67×10^{-4} M Tyramine labelled with C^{14} in a total of 1.5 ml of 0.05 M potassium phosphate buffer, pH 7.4. Platelet protein was assayed by the method of Lowry (Lowry, et. al.,1951), and platelet MAO was reported as nanamoles of deaminated product formed per milligram of platelet protein per 60 minutes of incubation (Roth, et. al., 1976).

RESULTS

Analysis of MAO activity correlation to age was not significant for the entire population of patients, family members of afflicted children and index patients. The lack of correlation was maintained for autistics, male autistics, family members alone, normals alone, and normals discriminated by sex. Sex differences revealed women (mean, 29.322) to have a significantly higher activity than males (mean,19.401).

Group means for normal males, male autistics and male family members were not significantly different. The sample size for autistic females was too small for adequate statistical analysis (Table 1). There was no significant difference between autistic males and normal males less than 24 years old (Table 2). MAO activity for normal males appeared to be normally distributed, though the distribution for male

autistics was not as clearcut (Figure 1).

MAO levels were assayed for parents, normal siblings and index children in nine families (Fig.2). Analysis of variance showed no significant clustering of MAO activity by family (F=1,313, NS).

DISCUSSION

Normative data to assess the relative contributions of age and sex on platelet MAO activity has been accumulated. Using BZ as a substrate, Robinson has studied a total of 71 men and 91 women, 21 to 84 years of age (Robinson,et. al., 1971; Robinson, 1975). He found a significant correlation with age in both populations, and that females have 27% higher MAO activity. It should be noted that the increase in activity is most pronounced after the age of 55, especially in women. A study of 680 normals through the age of 60 did not reveal any correlation with age in either sex, though females had activities 20% higher than males (Murphy, Wright, et. al., 1976). The frequency distribution significantly deviated from normal, resulting from a skewing of the curve by a few high values and more low values than expected. Statistical tests failed to reveal a bimodal distribution

Roth, Young and Cohen extended the normative data to children, with an eye to any influence of puberty on MAO

activity (Roth, Young & Cohen, 1976). The variability of MAO activity was 17.7% for all children, though it was 30% for females and only 10% for males. No correlation between age and activity was found through the age of 40 years. Females were found to have 23% higher activity at all ages. Prepubescent and pubescent females had 52% and 61% greater activity respectively. There was a significant decrease in activity in post-pubertal females. Nothing in the present study is in disagreement with the data previously reported.

This study corroborates previously reported work on MAO activity in autistic children (Boullin, et.al.,1975; Cambell, et. al.,1976; Cohen, Young & Roth, 1977; Lake, Ziegler & Murphy, 1977) showing no difference in activity from normals. The non-significant analysis of variance for activity in families reported here does not agree with the results of Cohen (Fig.2; Cohen, Young & Roth,1977). Cohen included two normal families in his study, and it is conceivable that the significant clustering he reported reflects a difference between normal and autistic families.

It appears that MAO does not contribute to the elevated 5-HT found in some autistic children. Simultaneous measurements of 5-HT and MAO would be most illuminating.

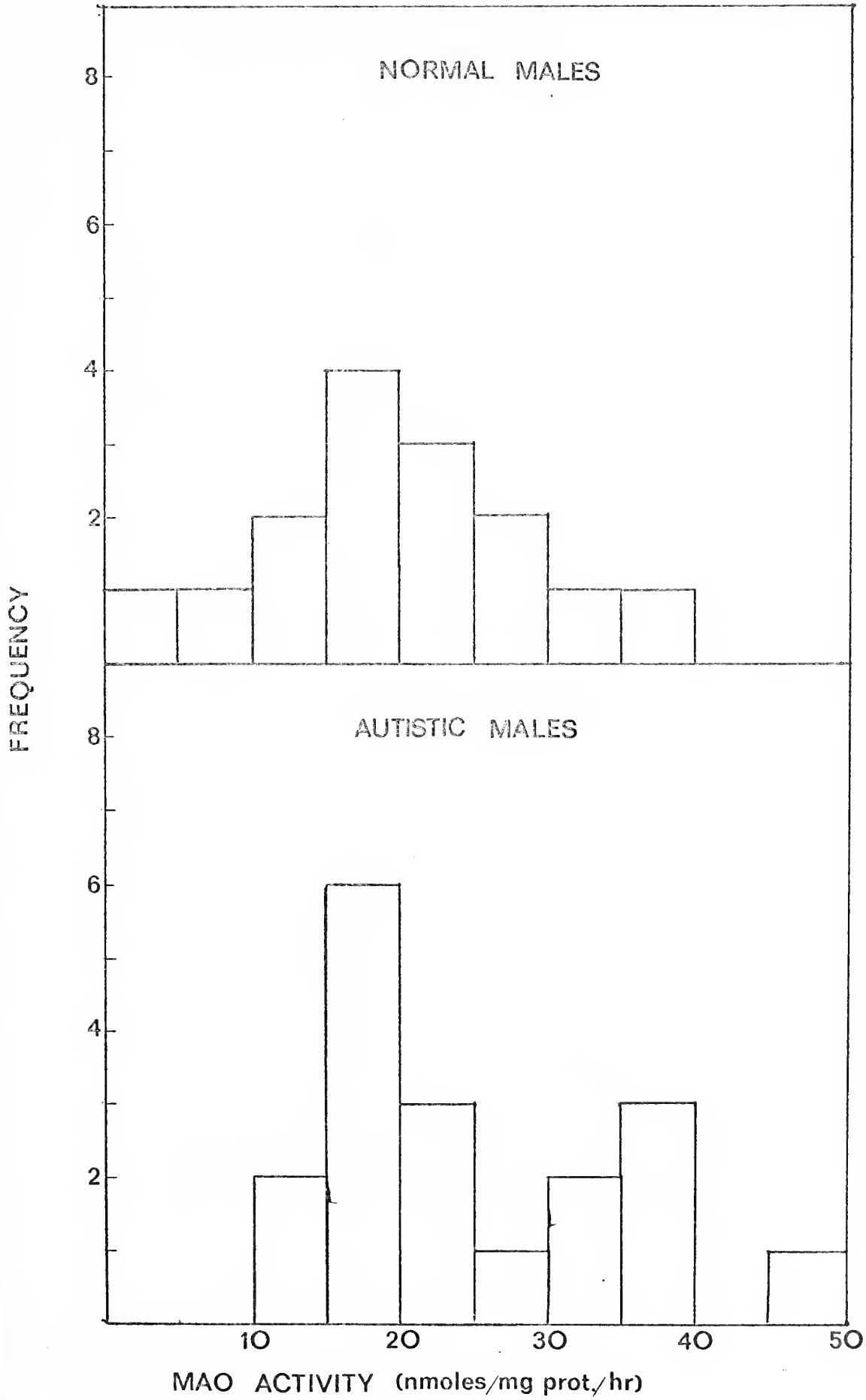
Table 1.- Group Means for Males

Platelet MAO Activity nmoles/ mg protein/ hr.			
Normal	Autistic	Family Member	P
19.401± 9.173 (N = 15)	24.814± 10.585 (N = 18)	**********	NS
19.401± 9.173 (N = 15)	********	22.974±8.459 (N = 12)	NS
**********	24.814±10.585	22.974±8.459	NS

Table 2.-Group means for Males, 24 years

Platelet MAO Activity nmoles/ mg protein/ hr		
NORMAL	AUTISTIC	P
23.1337±9.033 (N = 8)	24.8418±10.551 (N = 18)	NS

Fig. 1.- Distribution of MAO activity in Normal & Autistic Males



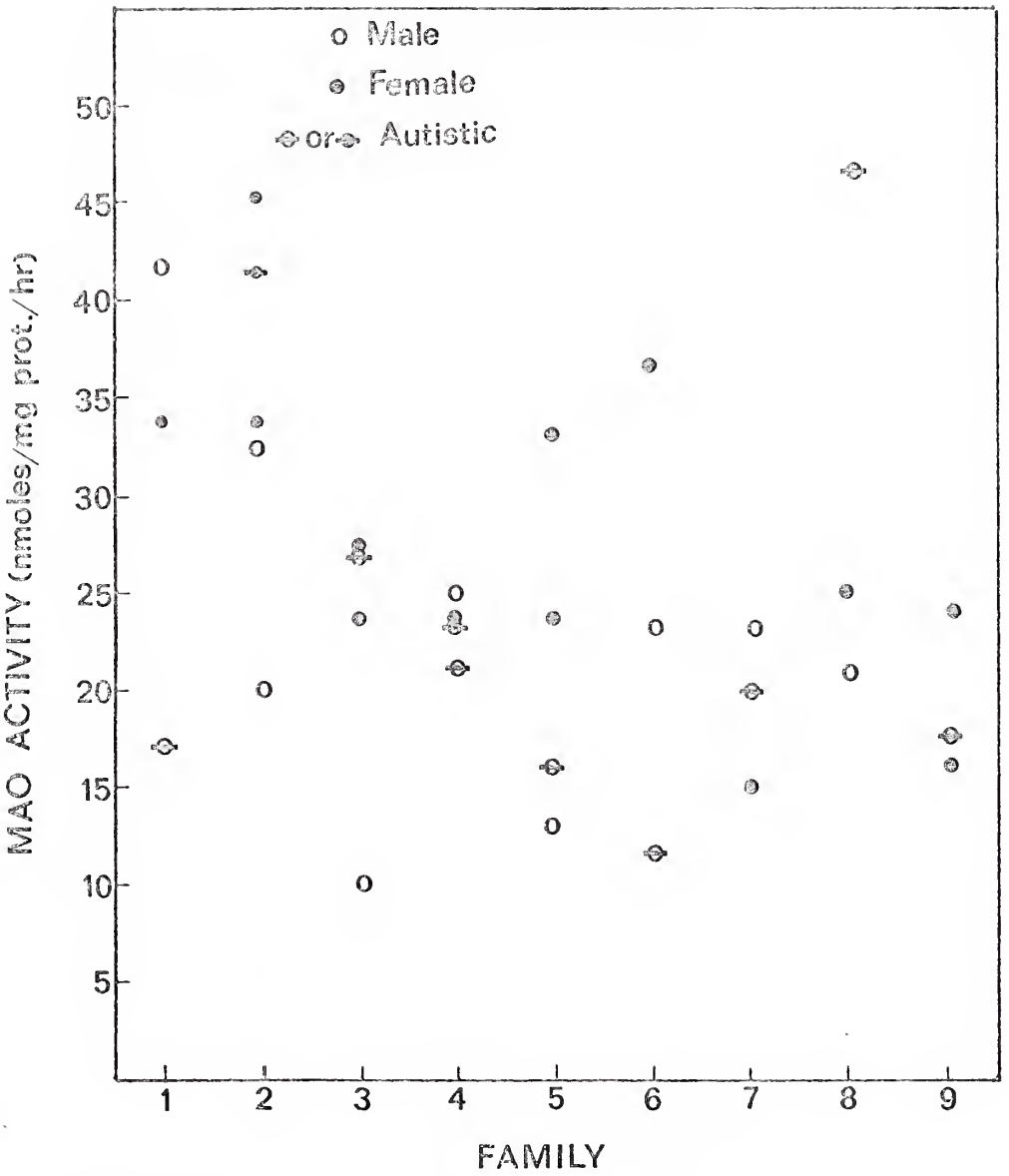


Fig. 2.- Platelet MAO activity grouped by families. For familial clustering, F=1.313; P=NS

BIBLIOGRAPHY

(1) Achee, F. M.,& Gabay, S. (1977): Biochem. Pharm,26:1637-1644

(2) _____, _____ & Tipton, K.F. (1977): Prog. in Neurobiology 8:325

(3) Blatchford,D., Holzbauer, M,& Youdim, M.B.H. (1975): Br. J. of Pharm. :251P

(4) Boullin,DJ, Bhagavan,HN, Coleman,M, O'Brien, RA & Youdim,MBH (1975): Medical Biology 53:210-213

(5) Bourgoin, S, Faivre-Bauman, A., Benda, P., Glowinski, J., Hamon, M. (1974): J. Neurochem. 23:319-327

(6) _____, Artaud, F., Adrien, J., Hery, F., Glowinski, J., Hamon, M. (1977): J. Neurochem 28:415-422

(7) Buchsbaum, MS, Coursey, RD, Murphy, DL (1976): Science 194:339

(8) Campbell,M; Friedman,E; Green,WH; Small,AM; Burdock,EI (1976): Neuropsychobiology 2:239-246

(9) Cohen,DJ; Young, JG; Roth, JA (1977): Arch. of Gen. Psych.34:534

(10) Collins,GGS; Sandler,M (1971): Biochem, Pharm. 20:289

(11) ____; Youdim,MBH; Sandler,M (1972): Bioch. Pharm. 21:1995

(12) Diaz-Borges,JM; D'Iorio,A (1972): Adv. in Bioch. Psychopharm. 5:79

(13)____;____ (1973): Canad. J of Bioch. 51;1089

(14) Donnelly, CH; Richelson,E; Murphy,DL (1976): Bio. Pharm.25:1639

(15) _____; Murphy,DL (1977): Bioch. Pharm 26:853

(16) Edwards, DJ; Burns,MO (1974): Life Sci 15:2045

(17) _____; Chang,S-S (1975): Bioch. & Biophys. Res. Comm.65:1018

(18) Eiduson,S (1972): Adv. in Bioch. Psychopharm. 5:271

(19) Ekstedt,B; Oreland,L (1975): Bioch. Pharm. 25: 119

(20)_____(1976): Bioch. Pharm. 25:1133

(21) Dennick,RG; Mayer,RJ (1977); Biochem. J. 161:167

(22) Edmondson,DE; Singer,TP (1976): FEBS Letters 64:255

(23) Fowler,CJ; Callingham,BA; Mantle,TJ; Tipton, KF (1978)
Bioch. Pharm 27:97

(24) Fuller, RW (1970): Bioch. Pharm. 19:2934

(25) Gabay, S; Achee, FM; Mentes, G (1976): J of Neurochem 27:415

(26) Gallagher, BM (1977) : Bioch. Pharm. 26:935

(27) Gear, ARL (1970): Biochem J. 120:577

(28) Ghosh, SK; Guha, SR (1978): Bioch. Pharm. 27:112

(29) Green, AR; Youdim, MBH (1975): Br. J. of Pharm. 55:415

(30) ____; Mitchell, BD; Tordoff, FC; Youdim,MBH (1977):
Br. J. of Pharm. 60:343

(31) Greenawalt, JW (1972): Adv. in Bioch. Psychopharm. 5:207

(32) Gripois,D (1975): Comp. Bioch. Physiol. 51c:143

(33) Guha, SR; Ghosh,SK (1970): Bioch. Pharm. 19:2929

(34) Hall, DWR; Logan,BW; Parsons, GH (1969): Bioch. Pharm. 18:1447

(35) Hartmann, BK; Udenfriend,S (1972): Adv. Bioch. Psychopharm.
5:119

(36) Hanley, HG; Stahl,SM; Freedman, DX (1977):Arch. Gen. Psy. 34:521

(37) Houslay,MD; Tipton,KF (1973): Biochem J 135:173

(38) _____;_____ (1975): Biochem. J. 145:311

(39) _____;____; Youdim,MBH (1976): Life Sci 19:467

(40) _____ (1977): J Pharm Pharmac 29:664

(41) Huang,RH; Eiduson,S; Shih,JC (1976): J of Neurochem 26:799

(42) _____; Eiduson,S (1977): J of Biol. Chem. 252:284

(43) Inoue F; Robinson,JB; Dost, KA (1976): J Pharm Pharmac 28:521

(44) Jain,M; Sands,F; Von Korff,RW (1973): Analyt. Bioch. 52:542

(45) _____;_____;(1974); J of Neurochem 23:1291

(46) _____ (1977): Life Sci 20:1925

(47) Johnston,JP (1968): Bioch. Pharm. 17:1285

(48) Kim,HC; D'Iorio, A (1968); Canad. J of Bioch.46:295

(49) Knoll,J; Magyar,K (1972): Adv. Bioch. Psychopharm 5:393

(50) Kobayashi,K; Eiduson, S (1977): Bioch. Medicine 18:378

(51) Kroon,MC; Veldstra, H (1972): FEBS Letters 24:173

(52) Lagnado,JR; Sourkes,TL (1968): Can.J. Bioch. Physiol. 36:587

(53) ____; Okamoto,M; Youdim,MBH (1971): FEBS Letters 17:117

(54) Lake,CR; Ziegler,MG; Murphy,DL (1977):Arch GenPsych 34:553

(55) Leckman, JF; Gershon,ES; Nichols,AS; Murphy,DL (1977)
Arch. Gen.Psych. 34:601

(56) Lowry,DH; Rosenbrough,MG; Farr,AL; Randall,RJ (1951)
J of Biol. Chem. 193:265

(57) Lyles,GA; Callingham,BA (1975): J Pharm Pharmac 27:682

(58) _____; Greenawalt, JW (1977): Bioch. Pharm. 26:2269

(59) Mantle,TJ; Wilson, K; Long,RF (1975a): Bioch. Pharm 24:2031

(60) _____;_____;_____(1975b): Bioch. Pharm 24: 2039

(61) _____;Houslay,MD; Garrett,NJ; Tipton,KF (1976):
J Pharm Pharmac 28:667

(62) ____; Garrett,NJ; Tipton,KF (1976): FEBS Letters 64:227

(63) McCauley,R; Racker,E (1973): Molec & Cell. Bioch. 1:73

(64) McEwen,Jr,CM; Sasaki, G; Lenz,Jr.,WR 91968:J BiolChem 243:5217

(65) _____;_____;Jones,DC (1969): Bioch, Easton 8:3952

(66) Meltzer,HY; Stahl,SM (1974):Res.Comm.Chem PathPharm 7:419

(67) Murphy,DL; Wyatt,RJ (1972): Nature 238:225

(68) ____; Weiss,R (1972): Am J of Psych 128:1351

(69) _____; Wright,C; Buchsbaum,M; Nichols,A; Costa,JL; Wyatt,RJ (1976) Bioch. Med. 16:254

(70) Neff,NH; Yang H-Y. T (1974): Life Sci 14:2061

(71) ____;____;Fuentes,JA (1974): Adv Bio Psychopharm 12:49

(72) Olivecrona,T; Oreland,L (1971): Biochemistry 10:332

(73) Oreland,L; Olivecrona,T (1971): Arch Bioch Biophys 142:710

(74) _____(1972): Adv Bioch Psychopharm 5:37

(75) Owen,F; Bourne,R; Crow,TJ; Johnstone EC; Bailey,AR; Hershon,HI (1976):Arch GenPsych 33:1370

(76) ____;____;Lai, JCK; Williams,R (1977):Bioch.Pharm 26:289

(77) Piszkiewicz,D (1977): Kinetics of Chemical and Enzyme-Catalyzed Reactions;pp.79-149;Oxford U. Press;NY

(78) Pletscher,A (1968): Br J of Pharm 32:1

(79) Robinson,DS; Lovenberg,W; Kaiser,H; Sjoerdsma,A (1968) Bioch.Pharm 17:109

(80) _____; Davis,JM;Nies,A; Ravaris,CL; Sylcester, D (1971) Arch. Gen. Psych. 24:536

(81) _____(1975): Fed.Proc. 34:103

(82) Roth,JA; Gillis,CN (1975): J.Pharm Exp Ther 194:537

(83) _____ (1976): J Neurochem 27:1107

(84) _____; Young, JG; Cohen, DJ (1976): Life Sci 18:919

(85) Rutter,M (1974): Psychol. Med. 4:147

(86) Sandler, M ; Youdim, MBH (1972): Pharmacological Reviews 24:331

(87) Schnaitman, C; Greenawalt, JW (1968): J of Cell Bio. 38:158

(88) Shih, JC; Eiduson, S (1974): Adv. Bioch. Psychopharm 12:29

(89) Sierens, L; D'Iorio, A (1970): Canad. J Bioch 48:659

(90) Singer, TP (1974): CIBA Found. Sympos.39(New Series):MAO and its Inhibitors; p.24; Elsevier,NY

(91) Sneddon, JM (1973): Prog. in Neurobiology 1:151

(92) Squires, RF (1972): Adv Bioch Psychopharm 5:355

(93) Stahl, SM (1977): Arch. Gen. Psych. 34:509

(94) Student, AK; Edwards, DJ (1977): Bioch. Pharm. 26:2337

(95) Sullivan,J; Stanfield, CN; Dackis, C (1977); Am. J of Psych 134:1098

(96) Tipton, KF; Spires, IPC (1968): Bioch. Pharm. 17:2137

(97) _____;(1971): in Mechanisms of Toxicity (W. N. Aldridge,ed.) pp.13-27; MacMillan,London

(98) _____(1972): Adv. Bioch. Psychopharm 5:11

(99) _____; Houslay, MD; Mantle, TJ (1976):CIBA Sym.39 (NS):MAO and its Inhibitors, pp.5-16

(100) Tooze, J (1973); Molecular Biology of Tumor Viruses; Cold Spring Harbor Laboratory, USA, pp.217

(101) Trendelenberg, U; Draskoczy, PR; Graefe,K (1972): Adv. Bioch. Psychopharm 5:371

(102) VandenBerg, CJ (1973):Metabolic Compartmentation in the Brain; (R. Balazs & J. E. Cremer,ed.),pp.137-166 MacMillan,London

(103) Van Woert, MH; Cotzias, GC (1966): Bioch. Pharm. 15:275

(104) Veryovkina, IV; Abdel Samed, M.M.; Gorkin,VZ (1972): Biochim Biophys Acta 258:56

(105) White, HL; Wu, JC (1975): J Neurochem 25:21

(106) _____; Glassman, AT (1977): J Neurochem 29:987

(107) _____; McLeod, MN; Davidson, JRT (1976): Am. J. Psych 133:1191

(108) Williams, CH (1974): Bioch. Pharm. 23:615

(109) _____; Lawson, J (1975): Bioch. Pharm. 24:1889

(110) Williams, D; Gascoigne, JE; Williams ,ED (1975). Brain Research 100:231

(111) Wyatt, RJ; Murphy, DL; Belmaker, R; Cohen, S;Donnelly, CH; Pollin, W (1973): Science 179:916

(112) Yang, H.-Y.; Neff,NH (1974): J Pharm Exp Ther 189:733

(113) Yasunobu, KT, Oi, S (1972): Adv. Bioch. Psychopharm 5:91

(114) Youdim, MBH; Sandler, M (1967): Biochem. J 105:43P

(115) _____; Collins, GGS; Sandler, M (1970):FEBS:SYMPOSIA 18- "Enzymes and Isozymes",D. Shugar,ed.;pp, 281-289 Academic Press; London

(116) _____;_____ (1971: Europ. J Bioch. 18:73

(117) _____(1974): Adv. Bioch. Psychopharm 11:59

(118) _____; Holzbauer,M; Woods, HF (1974): Adv. Bioch. Psychopharm 12:11

(119) _____; Collins, ggs (1975): ISOZYMES, Vol. II;ed. C.L. Markert, pps.619-636; Academic Press; NY

(120) _____; Holzbauer, M (1976): J. of Neural Transmission 38:193

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE
Marianne B. Powell EPH. 60 college St.	5/15/80.